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Effect of puromycin on metanephric differentiation: Morphological, autoradiographic and biochemical studies

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Effect of puromycin on metanephric differentiation: Morphological, autoradiographic and biochemical studies. Effect of aminonucleoside of puromycin (PAN) on metanephric development and proteoglycans (PGs) was investigated. Murine metanephric tissues, obtained on the thirteenth day of gestation, were exposed to PAN in a culture medium for one to seven days and processed for morphological, histochemical and immunofluorescent studies. For tissue autoradiographic and biochemical studies, kidneys were labelled with a precursor product of PGs, that is, [^{35}S]-sulfate. A generalized decrease in the glomerular population along with swelling and deformation in the ureteric bud branches was observed. These changes were accompanied with a diminution in the total incorporated radioactivity and a reduction in the autoradiographic grains, especially over the tips of ureteric bud branches. Sepharose CL-4B chromatography revealed a major high molecular weight PG ($M_r > 2.5 \times 10^6$), and a relative increase in the chondroitinase-ABC sensitive PGs. The media PGs were of relatively smaller size. Immunoprecipitation experiments with [^{35}S]-methionine-labeled tissues and immunofluorescent studies revealed a significant decrease of PGs in metanephric tissues, while type IV collagen and laminin were relatively unaffected. Significant glomerular changes included failure in differentiation of the visceral epithelial foot processes, formation of villi and in maturation of glomerular basement membrane. The latter was seen as fragments of extracellular matrices interspersed among undifferentiated podocytes and had reduced staining with ruthenium red—a dye marker for the PGs. This deficiency of PGs was confirmed by electron microscopic autoradiography, where a reduction in the number of silver grains was observed. The fact that the PAN-induced cellular and extracellular alterations were associated with perturbances in biosynthesis of PGs, suggests that the morphogenetic regulators, that is, PGs play a vital role in various differentiation processes involved during metanephric development.

The glomerulus undergoes a series of developmental stages during maturation which include: vesicle, S-shape body, precapillary and capillary [1–3]. In the precapillary stage, all three cell types of the glomerulus, although undifferentiated, can be recognized. At this stage, the undifferentiated glomerular podocyte lacks foot processes, and is seen as columnar epithelium forming occluding junctions with adjoining cells and investing the glomerular basement membrane from the outside. Such morphological features of the immature podocyte, that is, effaced foot processes and occluding junctions, are also seen in nephrosis induced by the administration of aminonucleoside of puromycin (PAN) [4–7]. According to some investigators, this change in the podocyte morphology may represent a step towards dedifferentiation during the induction of PAN nephrosis [1, 5]. Conceivably, the dedifferentiation may be a result of interference in cellular biosynthetic functions of the glomerulus. Indeed, PAN-induced biosynthetic alterations in certain connective tissue matrix glycoproteins have been observed in glomerular epithelial as well as renal organ culture systems [7–9].

During mammalian organ development, connective tissue extracellular matrices appear very early in embryonic life and conceivably they play a vital role in epithelial-mesenchymal interactions and morphogenesis [10, 11]. Such a morphogenetic role of extracellular matrices may also be applicable to renal organogenesis, particularly in glomerular maturation. Since certain connective tissue glycoproteins appear during various stages of glomerular development while others disappear, this would suggest their role in morphogenesis [12]. Among the various extracellular matrix proteins, laminin seems to appear in the early stages of glomerular development, as detected by immunohistochemical techniques [12]. At about the same time when epithelial-mesenchymal interactions are taking place, that is, S-shape body stage, there is usually a dramatic increase in the incorporation of precursor products of proteoglycans, that is, [^{35}S]-sulfate [3, 13, 14]. This means that more than one extracellular matrix glycoprotein appears during the critical stages of glomerular maturation.

The PGs have been shown to play a vital role in the processes of differentiation in various organs [14–23]. Along these lines, their role in glomerulogenesis has been elucidated in a metanephric culture system [14, 22]. In this organ culture system tremendous disturbances in nephrogenesis are observed in states of PGs deficiency. Here, presumably cellular synthesis of PGs either by epithelial or mesenchymal cells is perturbed, which ultimately results in poor maturation of the glomeruli. In view of the fact that PAN interferes in the epithelial cell metabolism, and conceivably induces dedifferentiation in vivo, we proceeded to investigate the effect of PAN on the sequence of events related to renal and glomerular morphogenesis with emphasis on PGs metabolism in a metanephric culture system.

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Methods

In this study, a metanephric culture system was employed, and various morphological, biochemical, light and electron microscopic autoradiographic techniques were used to elucidate the biosynthetic, cellular and extracellular matrical changes during renal development under the influence of PAN.

Organ culture system

Embryonic kidneys were maintained in an organ culture system as previously described [14, 22, 24, 25]. Briefly, Swiss-Webster (Charles-River, Wilmington, Massachusetts, USA) mice were allowed to mate in pairs in individual cages. Pregnant females were separated and maintained for 13 days after conception, following which the embryos were harvested aseptically and immersed in sterilized dishes containing a defined medium. The medium contained equal volumes of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (Sigma Chemical Co., St. Louis, Missouri, USA), supplemented with transferrin (50 μg/ml), penicillin (100 μg/ml) and streptomycin (100 μg/ml). In addition, HEPES was included in the media with a final concentration of 25 mM and pH maintained at 7.4. Metanephric tissues were dissected from the embryos and placed on the top of 8.0 mm filter discs (Millipore Co., Bedford, Massachusetts, USA) and floated on the serum free medium contained in a 35 mm petri dishes. Four to five metanephric kidneys (~1.0 mm in size) were placed on one filter paper disc. The kidneys were maintained at 37°C in an incubator with humidified 96% air and 4% CO_2 mixture.

Experimental design

The metanephric cultures were maintained for seven days and the kidneys examined on days 1, 4 and 7. Aminonucleoside of puromycin (PAN, 1 to 10 μg/ml) was included in the media, and the kidneys were exposed continuously for seven days. The PAN concentration of 2.5 μg/ml was found to be suitable for subsequent experiments since no discernible cytotoxic effects were observed, as ascertained by light and electron microscopy. At the end of the culture period the metanephric tissues (about 50 kidneys per time point per variable) were processed for morphological, histochemical and immunohistochemical studies as described below. For autoradiographic and biochemical studies, the PGs were labeled with isotopic precursor product [^{35}S]-sulfate (NEN products, Boston, Massachusetts, USA). Labeling was performed for 18 hours by inclusion of [^{35}S]-sulfate (specific activity > 1000 Ci/mmol) in the medium (0.5 mCi/ml) prior to harvesting of the culture. Both metanephric tissues as well as media were saved. To ascertain the effect of PAN on overall protein synthesis relative to PGs, a limited number of experiments were carried out with other isotopic precursor products, that is, [^{3}H]-leucine (0.25 mCi/ml) and [^{35}S]-methionine (0.25 mCi/ml), however, the labeling period was reduced to six hours.

Morphological studies

The metanephric tissues were scraped from the filter paper discs, immersion-fixed in Karnovsky's paraformaldehyde-glutaraldehyde fixative and processed for light and electron microscopy. For morphometric analysis, serial sectioning of the kidneys was performed. The sections (1 μm thick) in approximately to the center of the embryonic kidneys with inclusion of ureteric bud and its branching, were selected. They were stained with 0.1% toludine blue, photographed and printed to a final magnification of ×100. The number of glomeruli in various stages of development were enumerated. For day 1, the glomeruli in S-shaped body stage were examined. While for day 4, the glomeruli in both the S-shaped body and precapillary stages were studied. Finally, for day 7, only the precapillary stages were examined. Next, total area of the metanephric tissues was calculated by point counting method [14]. A transparent overlay, consisting of a calibrated grid with lines spaced 1 cm apart, was placed over the micrograph. Then the number of points encompassing the entire metanephric tissue was regarded as the relative area occupied by the embryonic kidney.

Histochemical studies

Histochemical studies were designed to ascertain the distribution of PGs present in the extracellular matrices during various stages of glomerular development. Ruthenium red (RR), a cationic dye was employed as previously described [2, 14] with certain modifications. The tissues were immersed in aldehyde fixative containing 0.2% RR and 1% cetylpridinium chloride (CPC) for three hours at room temperature. They were then immersed in 0.15 M cacodylate buffer and then post-fixed in 1% osmium tetroxide for four hours each; following which the tissues were processed for light and electron microscopy. The presence of RR as well as CPC in the post-fixative solutions appeared to be necessary for the visualization of RR-granules in the metanephric tissues.

Immunohistochemical studies

Next, the status of PGs which are specific to basement membranes, that is, heparan sulfate-proteoglycans (HS-PG), was investigated. Antibodies directed against the core peptide of HS-PG were employed; their isolation, purification and characterization have been described previously [3, 26]. Two micrometer thick serial sections were made by a cryostat and transferred onto glass slides. Sections which corresponded to the middle of embryonic kidney and included ureteric bud and its branches were selected. The sections were air dried, incubated with rabbit anti-HS-PG at 37°C for 30 minutes and washed with phosphate buffer saline (PBS). They were then incubated with FITC conjugated anti-rabbit IgG, followed by three PBS washes and examined under the ultraviolet microscope equipped with epi-illumination. The controls included staining of tissues with rabbit IgG only. To ascertain the status of other connective tissue proteins relative to PGs, the metanephric tissues were also stained with anti-type IV collagen and -laminin (provided by Dr. Dixit, Northwestern University) [27, 28].

Autoradiographic studies

A total of 150 kidneys, and 25 metanephric tissue samples per variable (CON vs. PAN) and time point (days 1, 4 and 7), were used for [^{35}S]-sulfate experiments. Subsequent to radiolabelling, the embryonic kidneys were washed with cold medium and immersed in the aldehyde fixative for three hours. They were then post-fixed in osmium tetroxide and processed for LM- and EM-autoradiography, as previously described [14, 29]. During these procedures, serial sections were made and sections representing the mid region of the metanephric tissue with the

urteric bud and its branches were selected. Approximately, 1 μm-thick sections, including the whole metanephric tissue, were processed for LM-autoradiography using either K5 or L4 emulsions (Polysciences, Warrington, Pennsylvania, USA). For EM-autoradiography, 60 nm-thick sections including various stages of glomerular development were processed.

Morphometric analyses were performed only on tissues processed for EM-autoradiography. Approximately 25 glomeruli, representing S-shaped body and precapillary stages, from each time point per variable were photographed. Each photograph included most of the individual glomerular stage within one EM-grid frame, and was printed to a final magnification of ×2500. Two compartments were designated: cellular and extracellular. Total number of grains were enumerated on each compartment by the best fit circle method of Salpeter and Bachman [30]. Next, the relative area of each compartment, was determined by point counting method described by Weibel [31]. The grain density (concentration of radiation) was calculated by dividing the total number of autoradiographic grains over a given compartment, by total area points. Finally, the mean grain density and standard deviation about the mean were determined.

The tissues from separate experiments with [^{3}H]-leucine and [^{35}S]-methionine were processed for LM-autoradiography only, their processing was essentially the same as described above.

Biochemical studies

Approximately 300 kidneys, with 50 metanephric tissues for each time point (days 1, 4 and 7) per variable (CON vs. PAN), were used in [^{35}S]-sulfate experiments for isolation and characterization of the PGs.

Extraction of metanephric tissue PGs was carried out as detailed in our previous publications [32, 33]. Briefly, the tissues were rinsed with cold medium and transferred to 4 M guanidine-HCl solution containing a mixture of protease inhibitors [10 mM 6-aminohexanoic acid, 5 mM bezamidine-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM diisopropyl fluorophosphate (DIFP), 10 mM sodium EDTA and 50 mM sodium acetate, pH 5.8]. Extraction was carried out for 24 hours at 4°C. The extracts were microfuged, supernatant saved and aliquoted. The sediment was hydrolyzed with 0.5 M NaOH at 60°C for two hours, thus solubilizing the remaining PGs/GAGs. Aliquots of supernatant and hydrolysate were applied to PD-10 column (Pharmacia Inc., Piscataway, New Jersey, USA), void volume fractions collected and total incorporated radioactivities determined. Values were normalized as per kidney. The remaining aliquots were dialyzed against distilled water containing 1 mM PMSF and DIFP, aliquoted, lyophilized, and stored at −20°C.

Characterization of extracted PGs was carried out by Sepharose CL-4B (Pharmacia Inc.) chromatography before and after treatment with chondroitinase-ABC (ICN Biomedicals, Costa Mesa, California, USA) or heparitinase (Sigma Chemical Co.), as previously described [34]. The molecular weight of PGs was estimated by comparing the Kav values of their elution peaks with the data obtained from enzymatic digests of cartilage PGs [35]. In a similar manner, characterization of glycosaminoglycans (GAGs), obtained after hydrolysis of PGs, was carried out. The molecular weights of the chains were calculated by comparison of Kav values of the elution peaks with the data obtained for chondroitin sulfate chains by Wasteson [36]. Further characterization of PGs/GAGs was carried out by DEAE-Sephacel (Pharmacia Inc.) chromatography using a gradient of 0.1 to 1.0 M NaCl in 8 M urea solution containing 50 mM sodium acetate and 0.2% (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS, Sigma Chemical Co.), pH 6.0 [14, 33]. Characterization of GAG chains, obtained after alkaline hydrolysis, were carried out by Sepharose CL-4B and 6B chromatographies before and after enzymatic treatments.

Characterization of the media PGs/GAGs was carried out as described above. For isolation of media PGs/GAGs, PD-10 (G-25 Sephadex) column were used. Void volume fractions were obtained, dialyzed, aliquoted, lyophilized and stored at −20°C. After reconstitution with 4 M guanidine or 8 M urea, the aliquots were subjected to Sepharose CL-4B and DEAE-Sephacel chromatography, and thus the characteristics of media PGs/GAGs determined.

In limited experiments with [^{3}H]-leucine or [^{35}S]-methonine, total incorporated radioactivities were measured as described above. In addition, immunoprecipitation was carried out with [^{35}S]-methionine labeled extracted products by using anti-PG, -type IV collagen and -laminin antibodies [26–28]. Thus, the incorporated [^{35}S]-methionine radioactivity in various matrix proteins was measured and compared to total radioactivity after normalization as per mg of protein or per kidney.

Results

Morphological features of metanephric tissues have been detailed in previous publications [14, 22]. Various stages of glomerular development which can be readily identified in the metanephric tissue include S-shape body and precapillary. At the thirteenth day of gestation (regarded as day 0 of metanephric culture), one can recognize glomeruli in S-shape body stage as well as a few in precapillary stage in the deeper regions of the cortex in close proximity to the tips of ureteric bud branches. Mature capillary stages, as seen in the neonatal rat or mice kidneys [1, 2] are not seen either on day 0 or 7 of the metanephric culture, as originally reported by Bernstein, Chen and Roszka [24] and confirmed by others [14, 22, 25]. Apparently, the vesicle stage is present on day 0, but it is difficult to recognize with certainity since it resembles the tubular segments in which no lumen has yet been formed. During the S-shape body stage, mesenchymal invasion occurs in the cleft region with further maturation into the precapillary stage. In the precapillary stage, one can observe mesangial and epithelial elements with concomitant development of extracellular matrices, such as the mesangial matrix and glomerular basement membrane (GBM). Endothelial cell lining is absent since metanephric culture is an in vitro organ culture system and no vascular invasion occurs in these tissues.

Morphological studies

In general, significant changes were observed in kidneys treated with PAN. All stages of glomerular development were affected to varying degrees. Minor reduction in the size of metanephric tissues was observed on day 1, but became noticeable on day 4, and was somewhat more pronounced by day 7. At the end of the organ culture period, metanephric tissues were reduced in their size by about 18%. In contrast to the mild decreases in the size of metanephric tissues, the glomeruli in

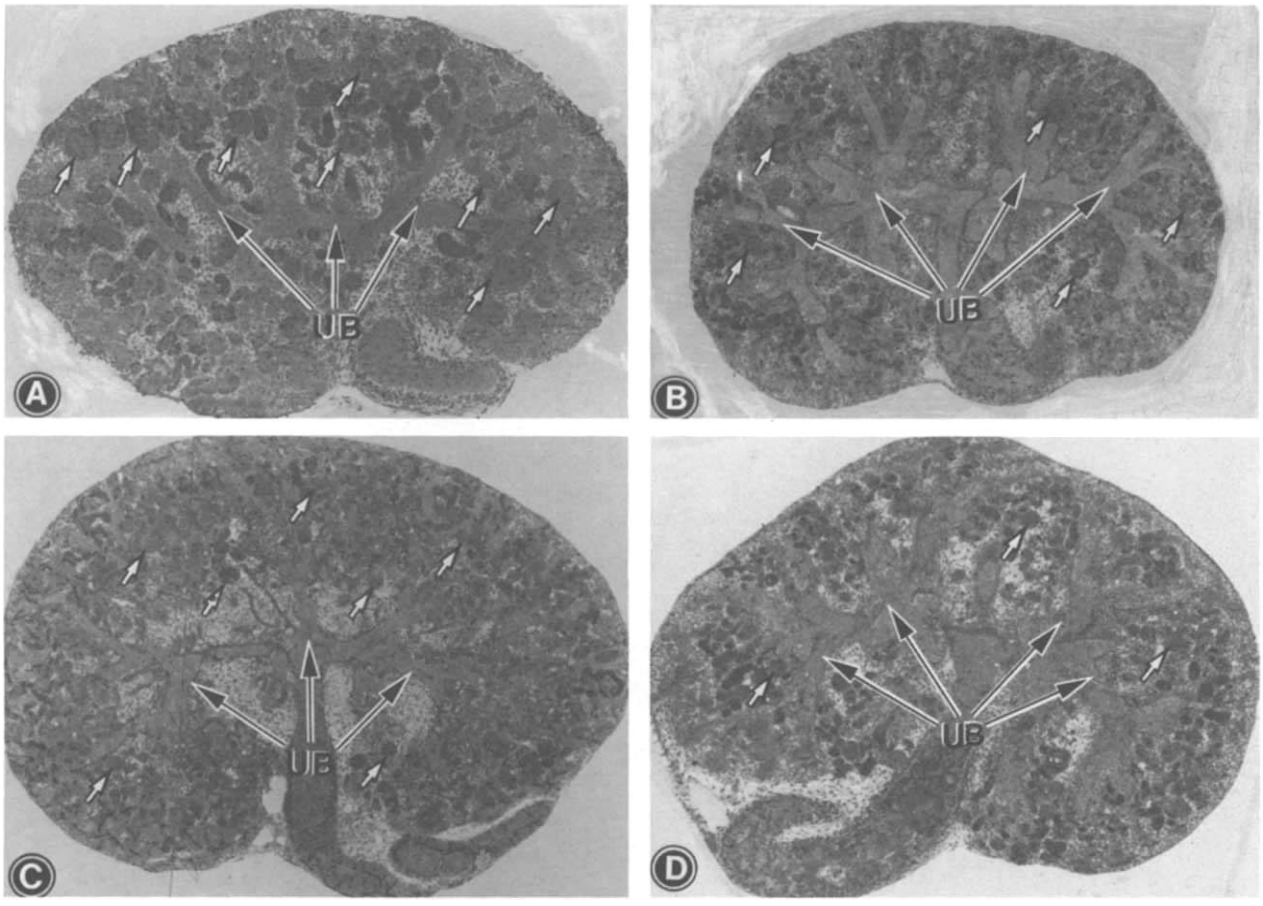

Fig. 1. *Light micrographs of control* **(A and C)** *and PAN-treated* **(B and D)** *metanephric kidneys after 1 (A and B) and 7 (C and D) days in culture.* At day 1 (A vs. B), there is a mild reduction in the population of the glomeruli (arrows), and swelling and deformation of ureteric bud branches (UB). These changes are pronounced after 7 days of PAN treatment (C vs. D). ×25.

Table 1. Total number of glomeruli and area of the metanephric tissues

	S-shape body		Precapillary	
	Number	Area	Number	Area
Day 1				
CON	19.50 ± 2.47	88.00 ± 4.81		
PAN	14.11 ± 3.51[a]	73.33 ± 7.10[a]		
Day 4				
CON	9.22 ± 1.78	130.20 ± 14.49	16.60 ± 2.19	130.20 ± 14.49
PAN	6.11 ± 1.36[a]	106.00 ± 6.85[a]	10.60 ± 1.14[b]	106.00 ± 6.85[a]
Day 7				
CON			32.50 ± 3.92	174.50 ± 23.12
PAN			16.83 ± 3.68[b]	142.16 ± 15.22[b]

Abbreviations are: CON, control; PAN, tissues treated with aminonucleoside of puromycin.
Values are expressed as mean ± SD; $N = 25$.
[a] Significantly different from the control ($P < 0.05$)
[b] Significantly different from the control ($P < 0.01$)

various developmental stages were relatively few. Even on day 1, a significant decrease of about 20% in the population of glomeruli in S-shape body stage was observed (Table 1). By day 4, further decrease in S-shape body stage glomeruli to about 34% was noted. Similarly, the number of glomeruli in precapillary stages on day 4 and 7 were reduced by ~36% and ~48%, respectively (Table 1). Thus, it appears that with elapse of culture period and PAN-treatment there was a disproportionate decrease in the population of the glomeruli as compared to the size of the embryonic kidneys. In addition to the decrease in the number of glomeruli, the ureteric bud and its branches were swollen, the latter exhibiting irregular ramifications. These changes could be seen on day 1 (Fig. 1A vs. 1B) but were much more pronounced on day 7 (Fig. 1C vs. 1D).

Next, an assessment of the changes in individual glomeruli at different passages of culture was made. At day 1, no discernible changes were observed. At day 4, mild reduction in the overall size of the glomeruli in the precapillary stage was observed. Other alterations included swelling of the visceral epithelial cells and diminished formation of their villi and extracellular matrices (Fig. 2A vs. 2B). These changes were quite distinct at day seven in both the cellular and extracellular (Fig. 2C vs. 2D) elements. In the controls, the extracellular matrices were well formed and a normal organization of visceral epithelial foot processes, flanking either sides of the glomerular basement membrane, was observed (Fig. 2C). In PAN-treated kidneys, the visceral epithelial cells (podocytes) exhibited poor develop-

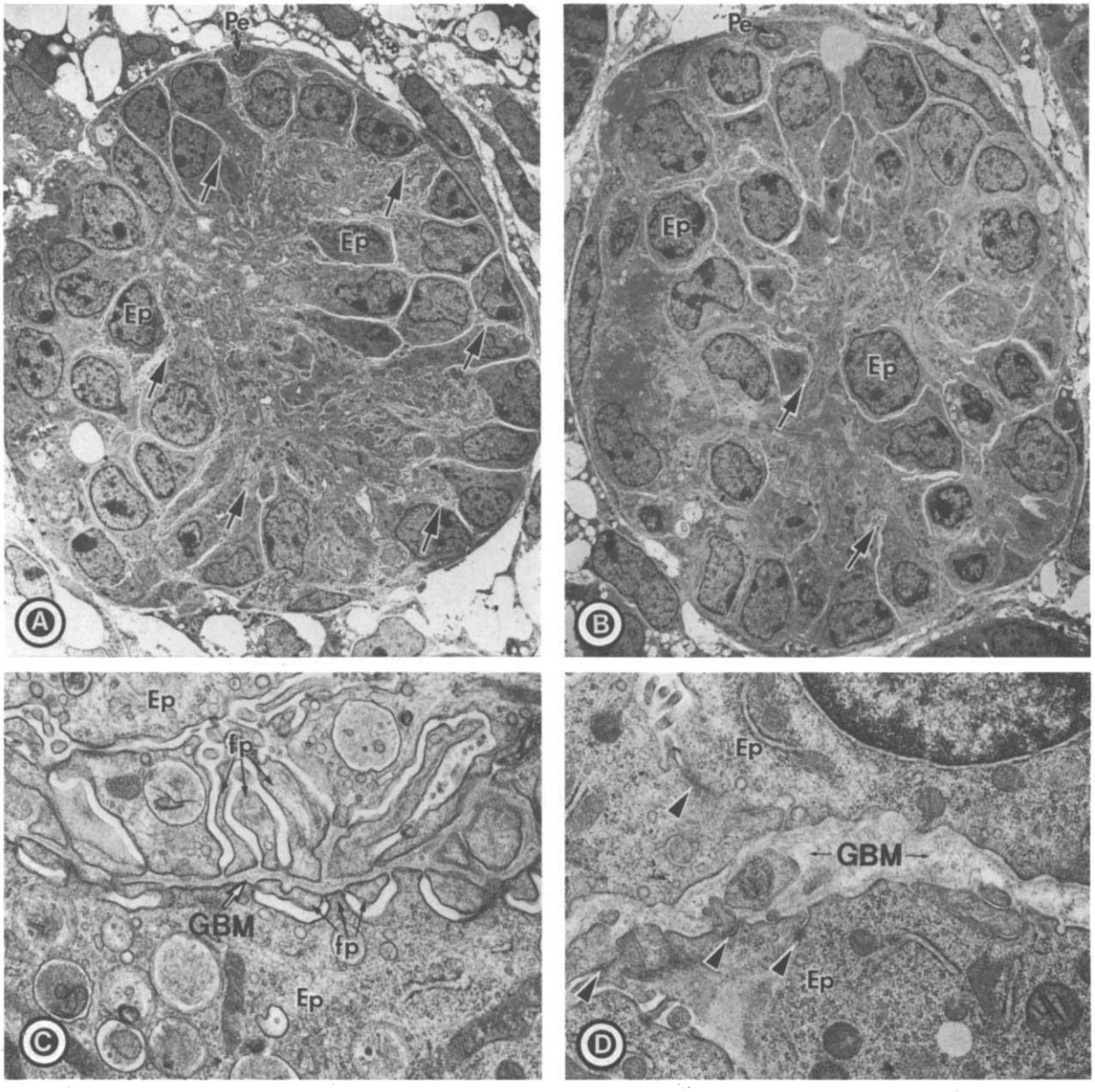

Fig. 2. *Electron micrographs of control* **(A and C)** *and PAN-treated* **(B and D)** *metanephric tissues after 4 (A and B) and 7 (C and D) days in culture.* At day 4, in precapillary stage, a reduction in the villi (arrows) of visceral epithelia (Ep) is observed (A vs. B). The parietal epithelia (Pe) are normal. At day 7, the glomerular basement membrane (GBM), foot processes (fp) and slit diaphragms of visceral epithelia (Ep) are poorly developed in tissues exposed to PAN (C vs. D). Also the occluding junctions (arrow heads) persist in PAN-treated tissues (panel D). A ×800; B ×1,000; C and D ×20,000.

ment of the foot processes and less compact organization of the GBM (Fig. 2D). Numerous intercellular occluding junctions persisted and aggregation of cytoskeletal elements in the podocytes, reminiscent of PAN nephrosis, was observed (Fig. 2D).

Histochemical studies

For these studies, the tissues stained with ruthenium red (RR), a cationic dye, were examined. The RR binds to protein-polysaccharide complexes (PGs) of extracellular matrices and they are seen as ~40 nm triangular or polyangular granules at the cell basal lamina interface or within the substance of the matrix [2]. By either fourth or seventh day of culture, significant changes were observed in the density as well as the size of the granules in both the S-shape body and precapillary stages of the glomeruli in metanephric tissues treated with PAN (Fig. 3). In the S-shape body stage, the matrices were poorly formed and had reduced number, as well as size of the RR-stained granules (Fig. 3A vs. 3B). In addition, the interconnecting filaments between individual granules were lost. Similarly, the RR staining was reduced or absent in the extracellular matrices of glomeruli in the precapillary stage (Fig. 3C vs. 3D). These histochemical alterations suggest an interference in the assemblage of extracellular matrix, perhaps related to defective

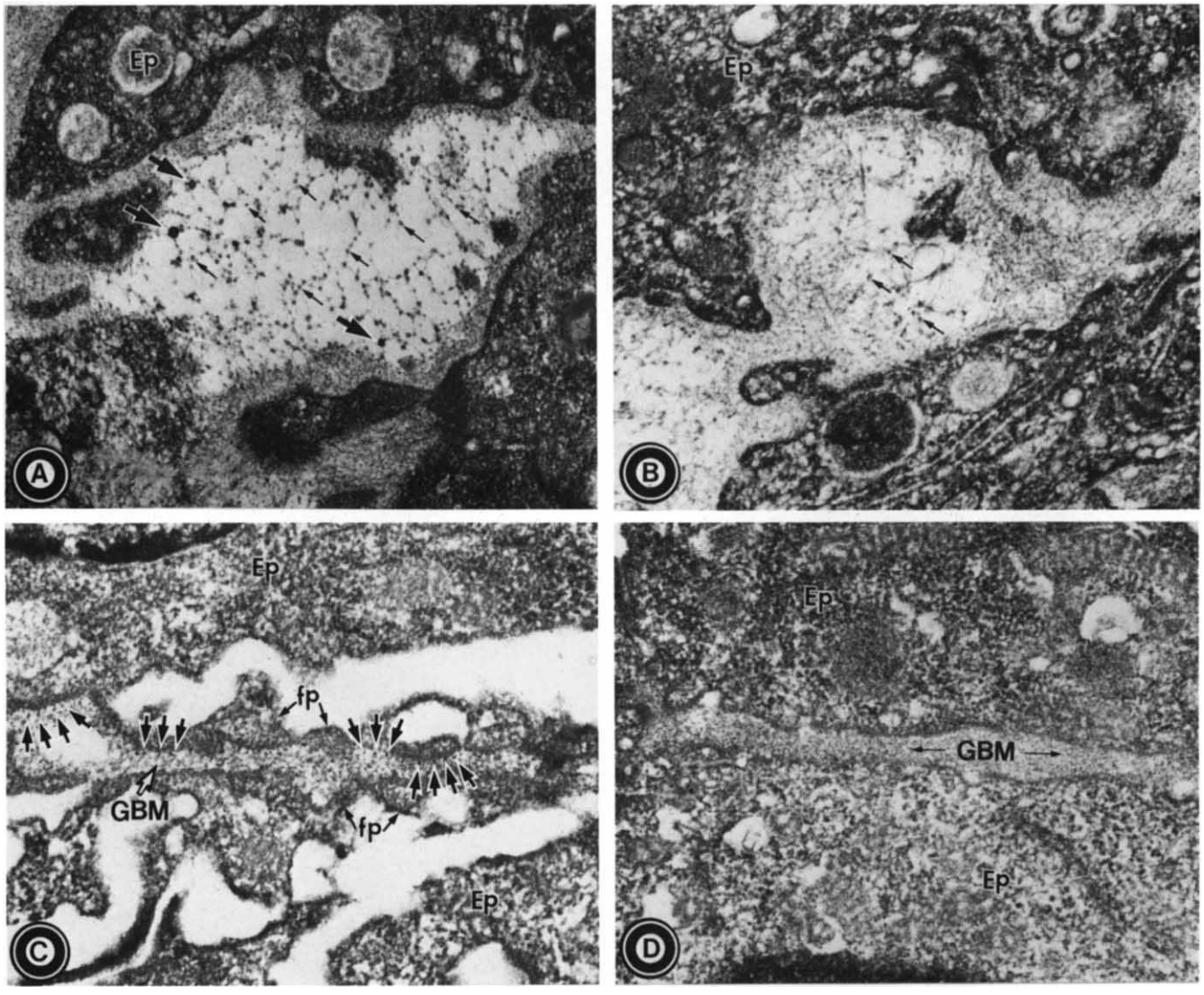

Fig. 3. *Electron micrographs of control* **(A and C)** *and PAN-treated* **(B and D)** *metanephric tissues after 4 (A and B, S-shape body stage) and 7 (C and D, precapillary stage) days in culture.* The tissues were stained with ruthenium red. In the cleft region of the S-shape body, a reduction in the size as well as in the number of ruthenium red granules (arrows) is observed (A vs. B). In addition there is a loss of interconnecting filaments between individual granules (A vs. B). In the precapillary stage of the control (panel C), the ruthenium red granules can be seen at periodic intervals (arrows) in the GBM; whereas their loss is noted after PAN-treatment (panel D). Abbreviations are: fp, foot processes; Ep, epithelium. A and B ×20,000; C and D ×15,000.

biosynthesis of PGs and epithelial-mesenchymal interactions under the influence of PAN.

Immunohistochemical studies

Antibodies directed against various components of the matrix, that is, proteoglycans, type IV collagen and laminin, were used. With the application of anti-PG antibodies, one could delineate basal lamina scaffolds investing the ureteric bud branches, glomeruli and tubules. On day 1, no differences were observed. With four days of exposure to PAN, mild reduction in the intensity of fluorescence was noted. On day 7, the differences in the staining were much more distinct. In addition to the sparse population of various nephron elements in the metanephric tissues, the glomeruli were smaller in size and their extracellular matrices had reduced reactivities with anti-HS-PG antibodies (Fig. 4A vs. 4B). The metanephric tissues stained with anti-type IV collagen did not reveal any significant differences between control and experimental groups (Fig. 5A vs. 5B). However, there was a mild decrease in the intensity of fluorescence of glomerular and tubular extracellular matrices of the metanephric tissues stained with anti-laminin (Fig. 5C vs. 5D).

Tissue autoradiographic studies

An overall decrease in the autoradiographic grains, although not distinct, was seen after one day of exposure to PAN (Fig. 6A vs. 6B). With continued PAN exposure, there was a further decrease in the incorporation, and by the seventh day obvious

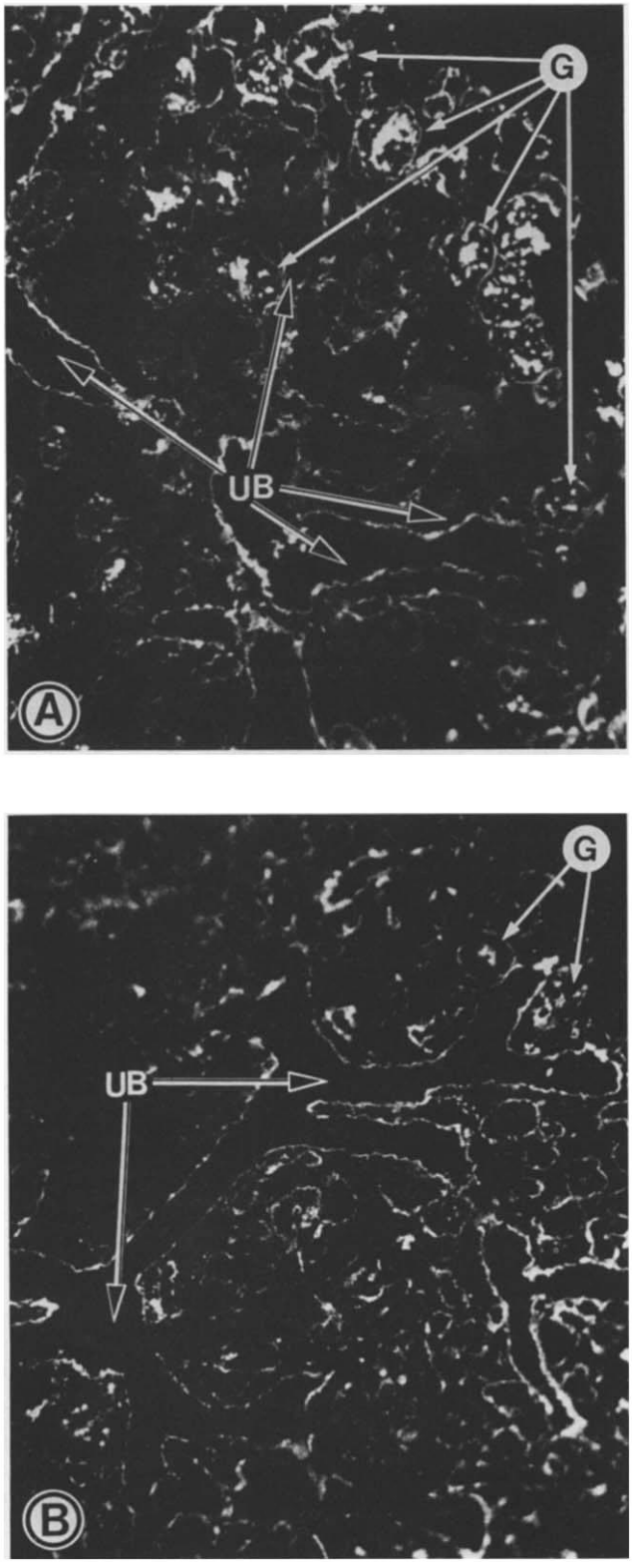

Fig. 4. *Indirect immunofluorescence micrographs of control* (**A**) *and PAN-treated metanephric tissues* (**B**) *after 7 days in culture, and stained with anti-HS-PG antibodies*. The control metanephric tissue is very well-populated with glomerular elements (G) and has well-organized ureteric bud branches (UB). In the PAN-treated tissues, there is a reduction in the population of glomeruli as well as a generalized decrease in the intensity of immunofluorescence. ×100.

differences were observed (Fig. 6C vs. 6D). The swollen ureteric bud branches as well as glomerular elements revealed relatively less silver grains outlining the respective extracellular matrices. To further ascertain alterations in the cellular and extracellular compartments and in different stages of glomerular development, EM-autoradiograms were processed and morphometric analyses performed.

In general, there was a decrease of grain densities in both intracellular and extracellular (matrix) compartments (Table 2). In the S-shape body stage, a minimal change in grain density was observed in the intracellular compartment at day 1. However, significant decreases in the grain densities were observed in the extracellular compartment. Such differences were not so evident in the cleft region of the S-shape body after four days of culture. On the other hand, in the precapillary stage, both the intracellular as well as extracellular compartments were affected (Fig. 7 vs. 8). A significant drop in the grain densities was noted with passage of the culture period in PAN-treated kidneys, which became quite evident by day 7 (Table 2). However, there were no significant differences in the number of grains present on the parietal epithelia and Bowman's capsular basement membranes (Fig. 7 vs. 8). In high magnification autoradiograms, a large number of grains were seen over the GBM, flanked on either side by well-differentiated visceral epithelial foot processes (Fig. 9B). Whereas relatively few autoradiographic grains were seen on the extracellular matrix of the glomeruli from kidneys treated with PAN (Figs. 9A and B vs. 9C). The reduction in the autoradiographic grains over poorly-developed extracellular matrices and visceral epithelial foot processes suggests that PAN has somehow perturbed the differentiation of the podocytes concomitant with the biosynthesis of the PGs.

Biochemical studies

With greater than 95% efficiency of extraction, an overall decrease in the total incorporated [^{35}S]-sulfate radioactivity was observed in the experimental groups compared to the controls (Table 3). Diminished incorporations were observed in both the tissue extracted as well as media fractions, commencing from day 1 and persisting throughout the culture period. However, maximal inhibition in the incorporation was observed at day 4 of culture with ~threefold decrease in both the cellular and media fractions (Table 3).

The metanephric tissue PGs were characterized by Sepharose CL-4B chromatography. On day 1, in the control group, a major peak (peak A) with Kav = 0.027 ($M_r > 2.5 \times 10^6$) was observed (Fig. 10A). Two minor peaks (peak B and C) with Kav = 0.38 ($M_r \sim 4 \times 10^5$) and 0.70 ($M_r \sim 3.0 \times 10^4$) were also noted. The peaks A and B contained large and a small sized PGs while peak C included GAG chains only. Treatment with chondroitinase-ABC released 30%, 50% and 50% of the radioactivities included in respective peaks A, B and C into the Vt fraction (Table 4), while treatment with purified heparitinase released 70%, 50% and 50% of the radioactivities from the respective peaks into the Vt fraction. Successive treatments with chondroitinase-ABC and heparitinase released all radioactivities associated with peaks into the Vt fraction. Elution profiles of PGs/GAGs, similar to those of the control, were observed in the experimental group on day 1, before and after enzymatic treatments (Fig. 10C). On days 4 and 7 in the control

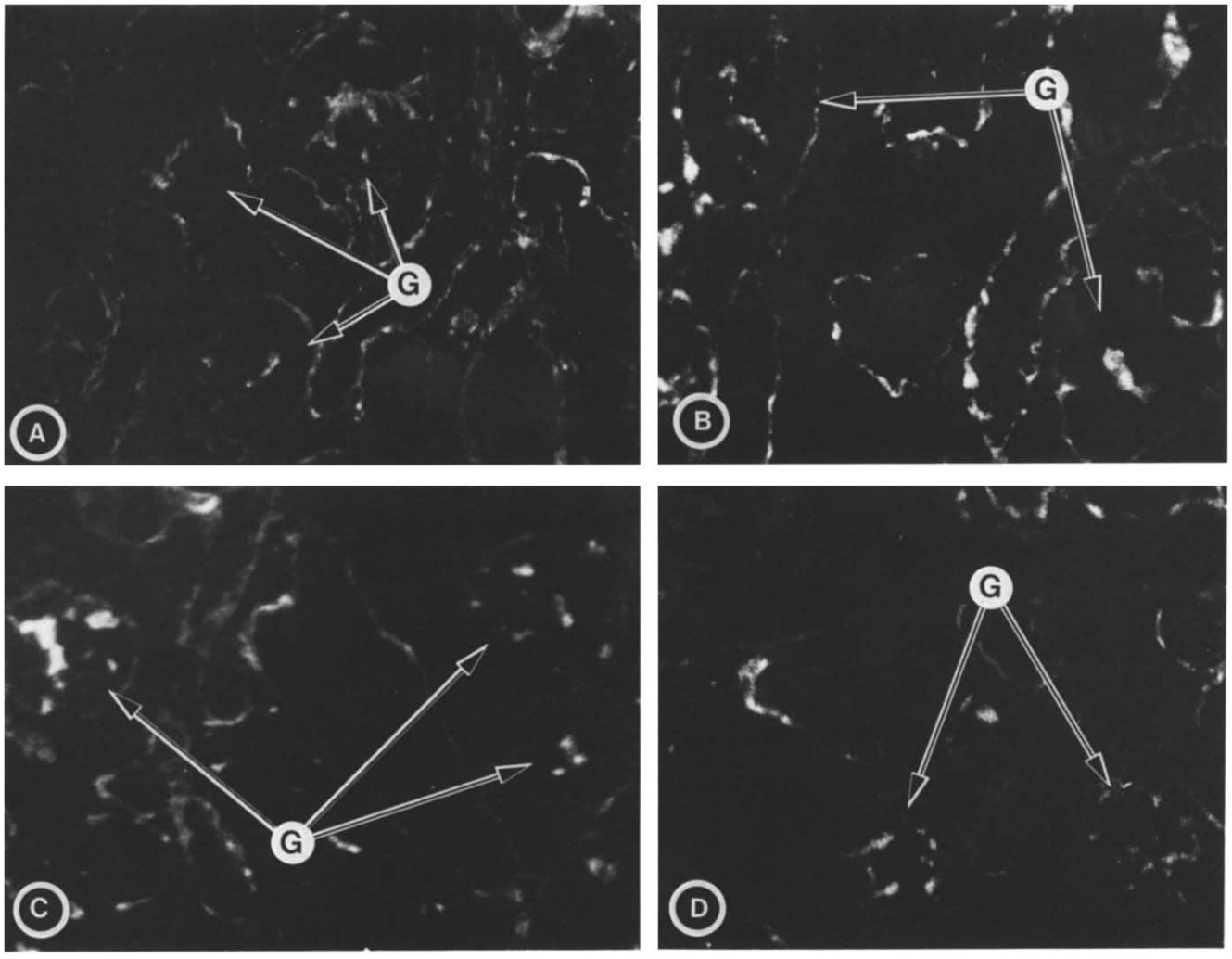

Fig. 5. *Indirect immunofluorescence micrographs of control* **(A and C)** *and PAN-treated metanephric tissues* **(B and D)** *after 7 days in culture, and stained with anti-type IV collagen (A and B) and -laminin (C and D) antibodies.* A mild reduction in the intensity of fluorescence is seen in the extracellular matrices of the glomeruli (G) from tissues exposed to PAN and subsequently stained with anti-laminin (C vs. D). ×200.

groups, relative proportions of incorporated radioactivities in peaks B and C increased, but the ratio of chondroitin sulfate to heparan sulfate essentially remained unchanged (Table 4). In the experimental groups, besides a generalized decrease in the incorporated radioactivity, the relative proportions of chondroitinase-ABC sensitive proteoglycans increased in the major peak, that is, peak A (Fig. 10B vs. 10D, Table 4).

The characterization of tissue GAGs by Sepharose CL-4B or CL-6B chromatographies also suggested a relative increase in the proportion of chondroitin sulfate, while the chain size remained the same. Estimated Kav values by Sepharose CL-4B and 6B were 0.68 and 0.42, respectively, with molecular weight $\sim 4 \times 10^4$.

The media PGs/GAGs, characterized by Sepharose CL-4B chromatography, revealed two peaks: a predominant peak A with Kav = 0.07 and a smaller peak B with Kav = 0.55 (Fig. 11A, Table 5). On day 1, the elution profiles were essentially similar between control and experimental groups. However, moderate increases in the chondroitinase-ABC sensitive PGs/GAGs were observed in both the peaks (Table 5). On day 7 in the control group, two peaks, a smaller peak A with Kav = 0.18 and a major peak B with Kav = 0.55, were observed. A relatively mild increase in the chondroitin sulfate was observed in both the peaks when compared to the elution peaks of day 1. In the experimental group of day 7, the elution peaks shifted to higher Kav values: peak A = 0.28 and peak B = 0.60 (Fig. 11B). Both the peaks had relatively higher proportions of chondroitinase-ABC sensitive PGs as compared to control day 7 (Table 5).

Further characteristics of tissue and media PGs/GAGs were carried out by DEAE-Sephacel chromatography. The tissue PGs/GAGs eluted between 0.4 to 0.5 M salt concentration of NaCl gradient, and no differences were observed between the control and experimental groups either at day 1 or 7 (Fig. 11C). The media PGs/GAGs at day 1 had a similar elution pattern as those of the metanephric tissues. However, at day 7 the media PGs/GAGs of the PAN group eluted at a relatively lower salt concentration as compared to the control (0.4 M vs. 0.5 M, Fig. 11D). In summary, the characteristics of PGs/GAGs synthesized under the influence of PAN are probably different from the control group.

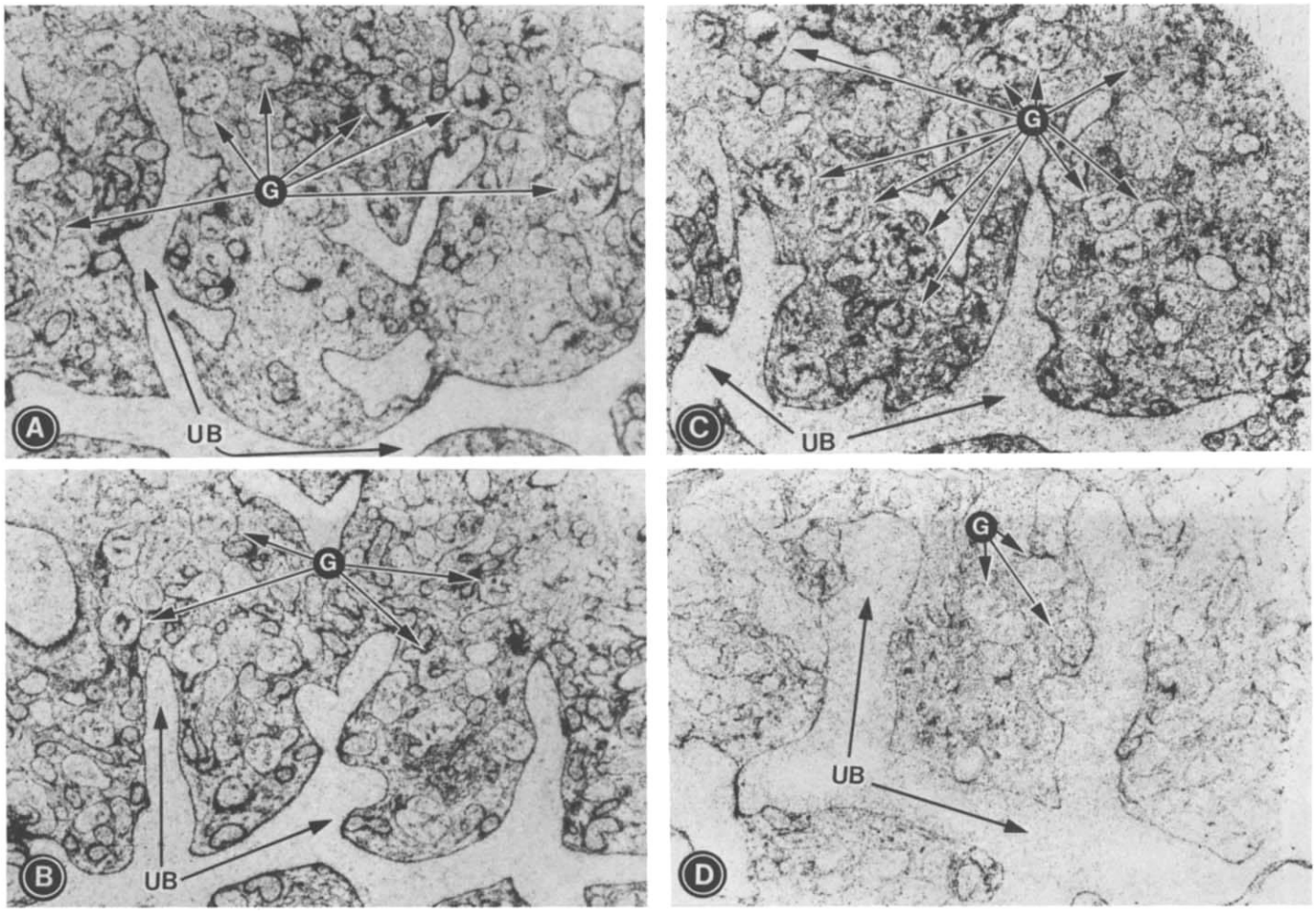

Fig. 6. *Light microscopic autoradiograms of control* **(A and C)** *and PAN-treated* **(B and D)** *kidneys taken from day 1 (A and B) and 7 (C and D) of the metanephric cultures.* At day 1, reduction in autoradiographic grains and population of the glomeruli (G), along with swelling and deformation of the ureteric bud (UB) branches are observed in PAN-treated kidneys. These changes are pronounced after 7 days of exposure to PAN. ×50.

Table 2. Total grains (TG), total area (TA) and autoradiographic grain densities (GD) of intracellular and extracellular compartments

	Intracellular			Extracellular		
	TG	TA	GD	TG	TA	GD
Day 1						
S-shape body						
CON	45.00 ± 2.19	91.83 ± 12.04	0.55 ± 0.08	143.16 ± 25.65	44.16 ± 7.17	5.52 ± 1.17
PAN	27.59 ± 5.16	50.01 ± 5.15	0.55 ± 0.07	91.83 ± 20.03	40.10 ± 6.44	1.99 ± 0.81[a]
Day 4						
S-shape body						
CON	33.50 ± 4.81	49.83 ± 4.41	0.60 ± 0.09	207.01 ± 20.97	40.20 ± 9.19	4.04 ± 0.71
PAN	29.66 ± 3.72	49.50 ± 8.19	0.59 ± 0.14	91.19 ± 21.29	25.00 ± 8.71	3.87 ± 1.76
Precapillary						
CON	51.80 ± 1.48	62.00 ± 6.04	0.81 ± 0.07	158.00 ± 17.88	31.20 ± 0.83	4.97 ± 0.49
PAN	34.01 ± 6.16	59.01 ± 5.34	0.53 ± 0.06[a]	107.04 ± 13.02	27.01 ± 4.36	3.84 ± 0.55[a]
Day 7						
Precapillary						
CON	48.91 ± 9.85	87.96 ± 9.84	0.57 ± 0.17	271.16 ± 47.16	47.16 ± 9.50	5.56 ± 1.94
PAN	26.25 ± 8.31	63.09 ± 7.70	0.48 ± 0.10	87.08 ± 25.55	44.50 ± 7.39	1.87 ± 0.68[a]

Abbreviations are: CON, control; PAN, tissues treated with aminonucleoside of puromycin.
Values are expressed as means ± SD; $N = 25$.
[a] Significantly different from the control ($P < 0.01$)

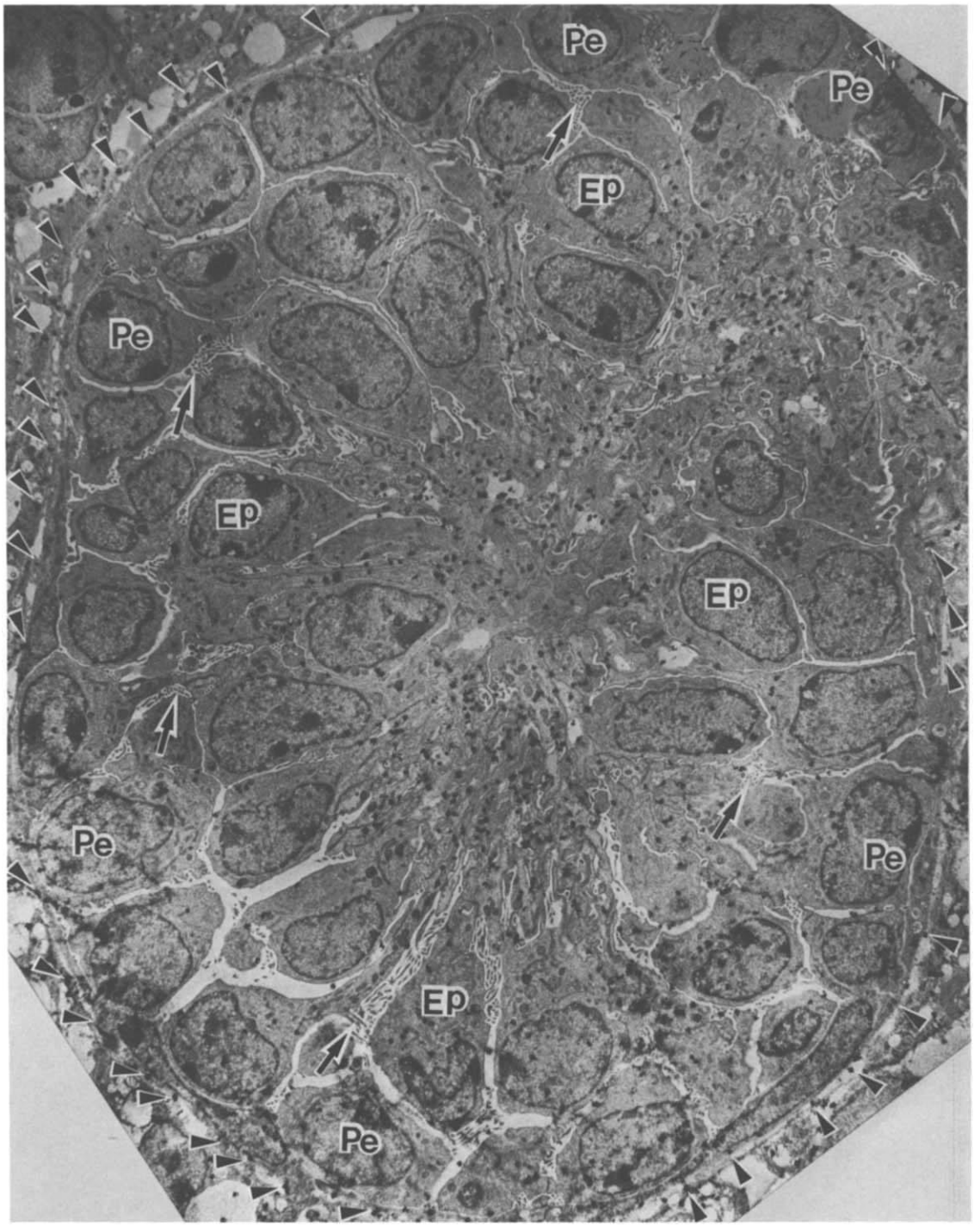

Fig. 7. *Low magnification electron microscopic autoradiograms of pre-capillary stage of control kidney labeled with [^{35}S]-sulfate after 4 days in culture.* Note the heavy concentration of autoradiographic grains in the central region where extracellular matrices are being developed. The visceral epithelial cells (Ep) have well developed villi (arrows). The silver grains are seen in a relatively higher concentration in the cytoplasm of visceral epithelial cell (Ep) as compared to the parietal epithelium (Pe). Also, a large number of grains are seen on the basal lamina scaffolds of Bowman's capsule (arrow heads). ×3,500.

[^{3}H]-leucine and [^{35}S]-methionine experiments

The results of these experiments revealed the status of overall protein synthesis. Total incorporated radioactivities were calculated after purification of extracts by PD-10 (Sephadex G-25) chromatography. A mild decrease in the [^{3}H]-leucine or [^{35}S]-methionine incorporation was observed in metanephric tissues treated with PAN for seven days (Table 6). To ascertain the level of incorporation in individual glomeruli, tissue LM-

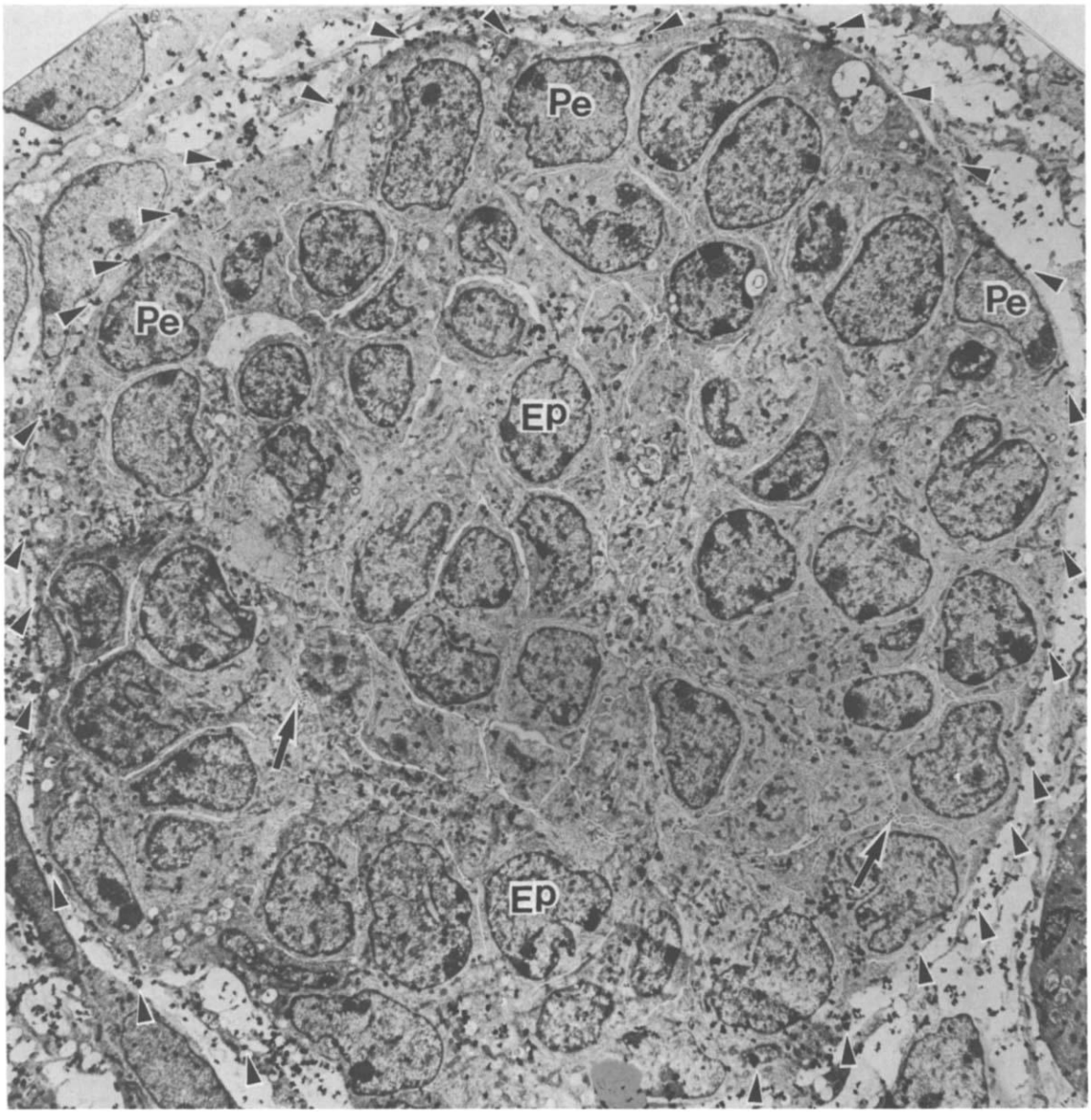

Fig. 8. *Low magnification electron microscopic autoradiogram of pre-capillary stage of PAN-treated kidney labeled with [^{35}S]-sulfate after 4 days in culture.* A generalized reduction in autoradiographic grains over cellular as well as extracellular compartments of the glomerulus is observed. A usual number of grains is seen on basal lamina of the Bowman's capsule (arrow heads). The visceral epithelium (Ep) has less number of villi (arrows). The parietal epithelium (Pe) is normal. ×3,000.

Table 3. Total radioactivity in tissues and media

	Tissue 10^5 dpm/kidney[a]			Media 10^5 dpm/ml[a]		
	Day 1	Day 4	Day 7	Day 1	Day 4	Day 7
CON	~1.06	~3.81	~3.21	~2.74	~4.21	~5.71
PAN	~0.51	~1.35	~1.84	~1.68	~1.49	~2.80

Abbreviations are: CON, control; PAN, tissues treated with aminonucleoside of puromycin.

[a] Twenty-five kidneys were used per variable and pooled for extraction

Table 4. Percentage radioactivity in various peaks of metanephric tissue proteoglycans

	Day 1	(CS	HS)	Day 4	(CS	HS)	Day 7	(CS	HS)
CON									
Peak A	~80	(30	70)	~53	(30	70)	~57	(30	70)
Peak B	~10	(50	50)	~25	(50	50)	~28	(50	50)
Peak C	~10	(50	50)	~22	(50	50)	~15	(50	50)
PAN									
Peak A	~80	(30	70)	~43	(50	50)	~56	(50	50)
Peak B	~10	(50	50)	~38	(50	50)	~24	(50	50)
Peak C	~10	(50	50)	~19	(50	50)	~20	(50	50)

Abbreviations are: CON, control; PAN, tissues treated with aminonucleoside of puromycin; CS, chondroitin sulfate; HS, heparan sulfate.

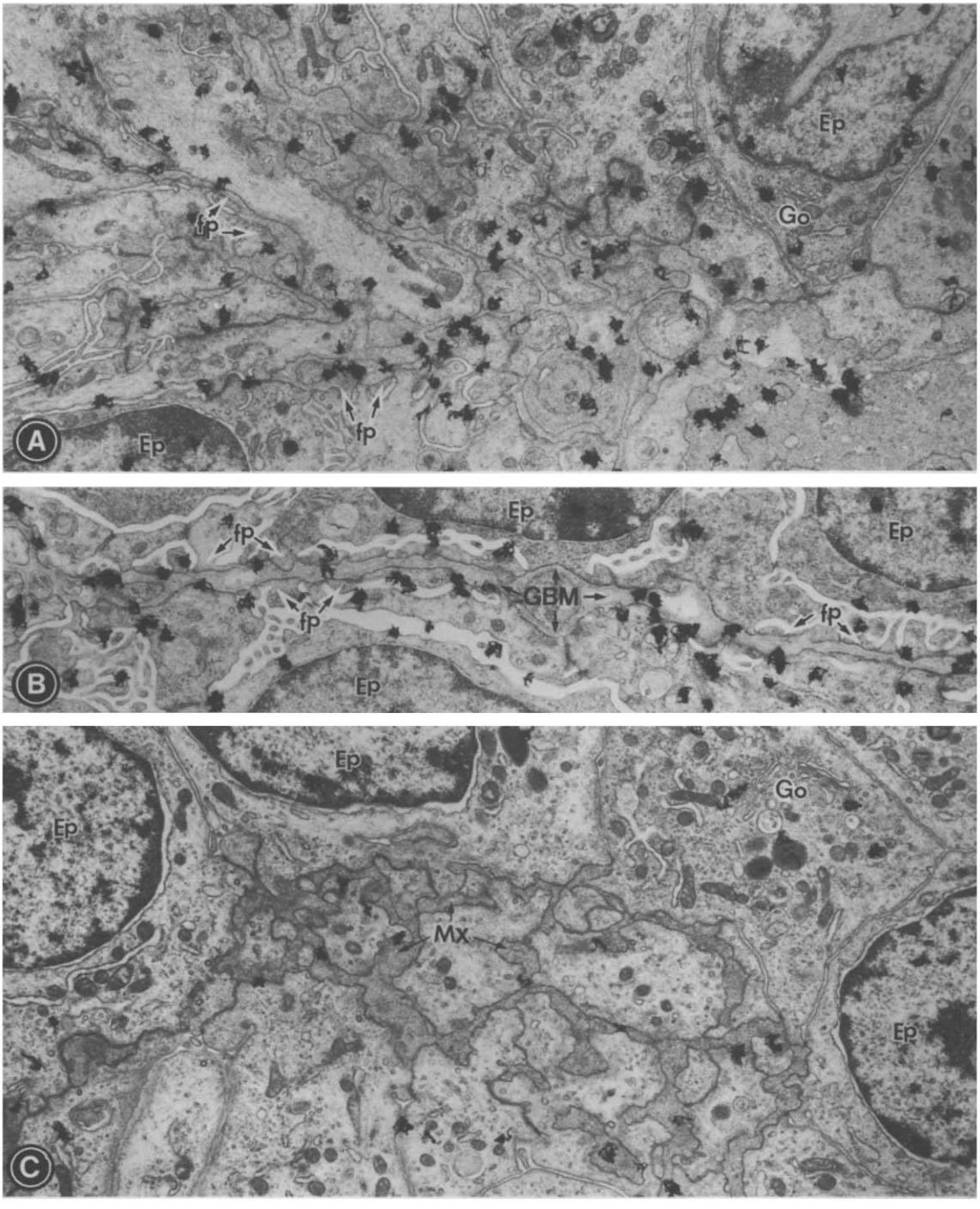

Fig. 9. *High magnification electron microscopic autoradiograms of pre-capillary stage of control* **(A and B)** *and PAN-treated kidneys* **(C)** *labeled with [^{35}S]-sulfate after 7 days in culture.* In control autoradiograms large number of silver grains associated with the glomerular basement membranes (GBM) are seen. The foot processes (fp) of the visceral epithelium (Ep) are well developed. In the PAN-treated kidney, the basement membranes are not formed and fragments consisting of loose matrix (Mx) are seen interspersed among the undifferentiated epithelia. There are fewer silver grains associated with cellular and matrical elements. The Golgi apparatus (Go) appears to be normal. A and B ×12,500; C ×10,000.

autoradiograms were prepared. No significant differences in the radioincorporation of either [^{35}S]-methionine (Fig. 12A vs. 12B) or [^{35}S]-leucine (Fig. 12C vs. 12D) were observed between the control and experimental groups. The immunoprecipitation experiments with anti-laminin and -type IV collagen showed mild decreases in the radioactivities of respective extracellular matrix proteins (Table 7), whereas a notable reduction, ~3-fold in the radioincorporation into the HS-PG, was observed. This

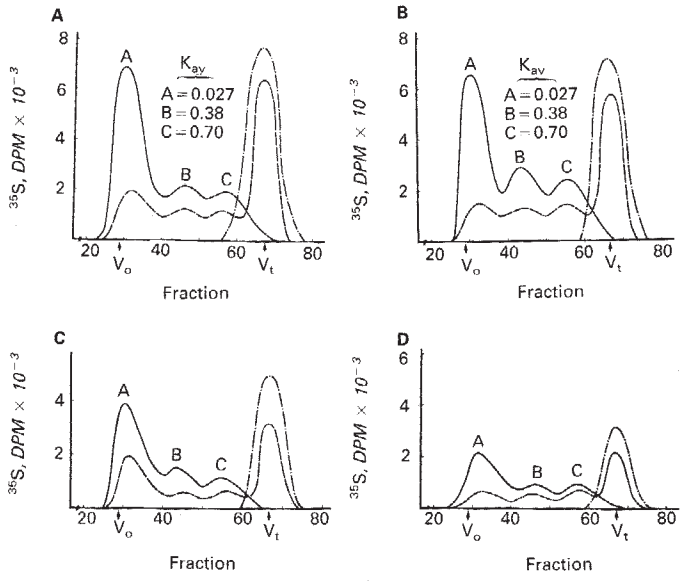

Fig. 10. *Sepharose CL-4B chromatograms of [^{35}S]sulfate-labeled proteoglycans extracted from embryonic kidneys after 1* **(A and C)** *and 7* **(B and D)** *days in cultures.* Panels A and B represent control while C and D are from kidneys treated with PAN. On day 1 (A and C), the intact PGs (—) eluted as three peaks. Treatment with purified heparitinase (- -) released 70%, 50% and 50% radioactivities from peaks A, B and C into the Vt fraction; while further treatment with chondroitinase-ABC released 100% of the radioactivity from all the peaks into the Vt fraction (- · -). In control, day 7 (B) peaks B and C are accentuated but the proportion of heparan and chondroitin sulfates remains the same. In kidneys treated with PAN for 7 days (D), the radioactivity associated with all the peaks is remarkably reduced and there is a relative decrease in the heparitinase-sensitive PGs in peak A.

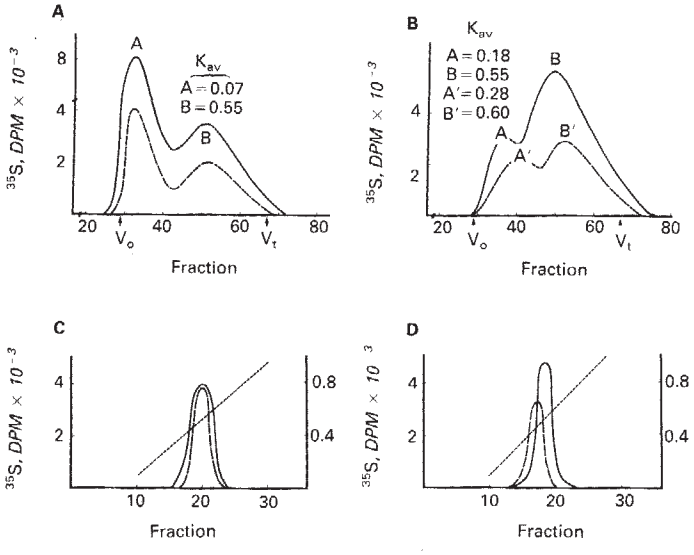

Fig. 11. *Sepharose CL-4B* **(A and B)** *and DEAE-Sephacel* **(C and D)** *chromatograms of [^{35}S]-sulfate labeled media PGs/GAGs from day 1 (A and C) and day 7 (B and D) of metanephric cultures.* On day 1 (A and C), no differences are observed between control (——) and PAN-treated (- -) kidneys except for reduction in the total radioactivities associated with various peaks. On day 7, Sepharose CL-4B chromatograms show peak shifts (A → A′, B → B′) to smaller molecular weight PGs in PAN-treated (- -) kidneys when matched with controls (——). Also, on day 7, DEAE-Sephacel chromatograms show that PGs from PAN-treated (- -) kidneys elute at a relatively lower salt concentration as compared with the control (——).

Table 5. Percentage radioactivity associated with chondroitin sulfate (CS) and heparan sulfate (HS) in various peaks of media proteoglycans/glycosaminoglycans

	Day 1		Day 4		Day 7	
	CS	HS	CS	HS	CS	HS
CON						
Peak A	~50	~50	~55	~45	~60	~40
Peak B	~44	~56	~50	~50	~54	~46
PAN						
Peak A	~60	~40	~68	~32	~70	~30
Peak B	~54	~46	~60	~40	~60	~40

Abbreviations are: CON, control; PAN, tissues treated with aminonucleoside of puromycin; CS, chondroitin sulfate; HS, heparan sulfate.

Table 6. Effect of PAN on [^{3}H]-leucine and [^{35}S]-methionine incorporation

	[^{3}H]-leucine	[^{35}S]-methionine
	dpm/kidney[a]	
CON	~3.54 × 10^5	~1.07 × 10^7
PAN	~3.18 × 10^5	~0.86 × 10^7

Abbreviations are: CON, control; PAN, tissues treated with aminonucleoside of puromycin.

[a] Twenty-five kidneys were used per variable and pooled for extraction

Table 7. Immunoprecipitation data of [^{35}S]-methionine labeled tissue extracts

	Control	PAN-treated
	dpm/kidney	
Normal rabbit serum	~180	~198
Anti-HS-PG core protein	~8780	~2850
Anti-laminin	~9080	~7230
Anti-type IV collagen	~4260	~3870

Abbreviations are: PAN, tissues treated with aminonucleoside of puromycin for 7 days; HS-PG, heparan sulfate-proteoglycan.

[a] The experiments were carried out in triplicates

suggests the possibility that PAN selectively affected the biosynthesis of PGs in the metanephric culture system.

Discussion

The results of this investigation indicate that PAN induces morphological and biochemical alterations in both the cellular and extracellular elements during renal morphogenesis. The changes included reduction in the population of nephron elements, swelling of the ureteric bud branches with irregular ramifications and reduced [^{35}S]-sulfate incorporation. The specific glomerular alterations were in the form of poor maturation of the podocytes, failure in the development of foot processes and loose organization of the GBM along with deficiency of PGs.

The cellular changes in the glomeruli resemble those seen in PAN nephrosis [4–7]. The notable changes observed in PAN nephrosis include fusion of the foot processes, development of inter-epithelial occluding junctions [4–7] and possible loss of certain antigenic epitopes of HS-PGs [37]. As indicated earlier,

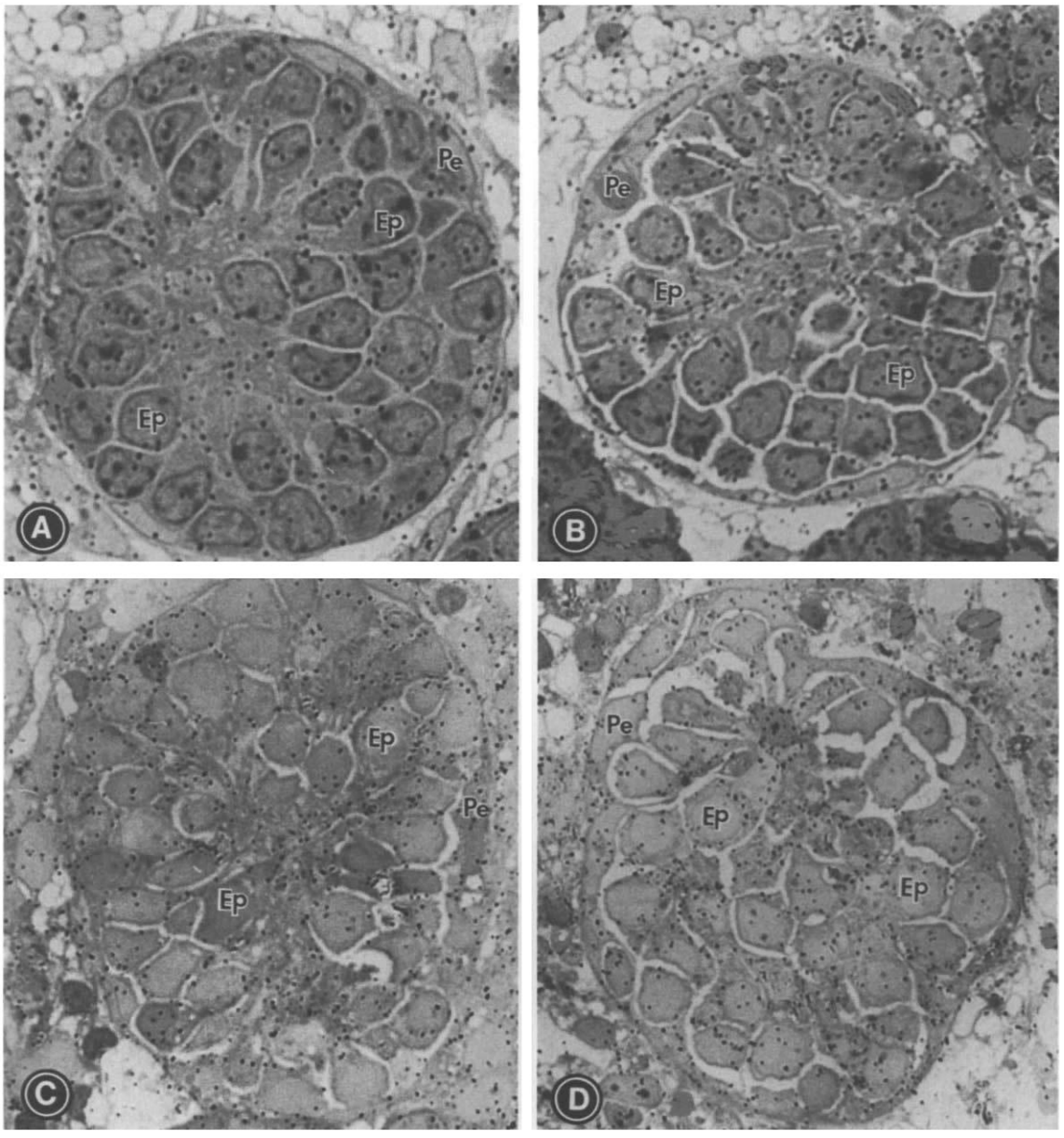

Fig. 12. *Light microscopic autoradiograms of metanephric tissues (precapillary stage) labeled with [^{35}S]-methionine* **(A and B)** *and [^{3}H]-leucine* **(C and D)** *after 7 days in culture.* The panels A and C represent control and B and D are from metanephric tissues treated with PAN. No obvious differences are observed between control and PAN-treated tissues. The variability in the size of autoradiographic grains, that is, between A and B vs. C and D, is related to the differences in radiation spread of the isotopes. ×500.

such changes are more or less regarded as dedifferentiation of the glomerulus since these characteristics have been observed in immature glomeruli [1–5]. Also, in an immature glomerulus the GBM is not well developed and lacks the organization of RR staining granules [2]. Taking into account the cellular and extracellular changes observed here, one can say that PAN indeed has inhibited the differentiation, and it also interfered in the metabolism of proteoglycans.

The interference in the PGs metabolism is further reflected by the autoradiographic and biochemical studies. The reduction in the autoradiographic grain density (concentration of radiation) was noted in both the cellular and extracellular compartments. Such changes have been observed in other organ systems [16, 19] as well as in renal cultures [14] where morphogenetic events have been investigated. The fact that the autoradiographic grain density was reduced in the inception stages of development, that is, in the cleft region of S-shape body, indicates that perturbed biosynthesis of PGs may have adversely affected further maturation of the glomerulus. Unique to this stage of glomerular development are critical epithelial-mesenchymal interactions proceeding in the cleft region, therefore an optimal concentration of PGs is perhaps necessary to sustain morpho-

genesis. Any interference in the biosynthesis of PGs would be expected to result in poor glomerular development, and findings similar to those observed here were noted in our earlier experiments with xyloside [14]. In addition to the glomerular developmental changes, autoradiographic densities were also reduced over the disorganized ramifications of the swollen ureteric bud branches, especially in their distal tips, the sites where nephrons develop. This decrease in the PGs perhaps resulted in the poor budding of the nephrons which ultimately was reflected in a generalized population decrease of various nephron elements of the metanephric tissue. Thus, it appears that the PGs are necessary not only for the glomerular morphogenesis but also for organogenesis of the kidney as a whole. In any event, critical to the sustenance of morphogenesis/organogenesis are proper epithelial-mesenchymal interactions in vital regions of a given organ or its various parts. Such vital epithelial-mesenchymal interactions in selective regions of other organs have been shown to play a critical role in their morphogenesis. For instance, in salivary gland cultures one finds very high concentration of PGs in the cleft region of the lobule relative to that in the peripheral advancing tips [16, 19, 38]. This differential along with active epithelial-mesenchymal interactions have been regarded as necessary to maintain lobulogenesis of the salivary gland. It has been shown that perturbance in the synthesis of PGs, or abolition in the concentration gradient (between the tips and cleft) or interference in the epithelial-mesenchymal interactions results in failure of the lobulation [16, 19, 38]. Presumably, more or less similar interactions take place in other organs where morphogenesis have been investigated [11]. Thus, the present findings reinforce the biological importance of the role of PGs in morphogenesis.

Morphogenesis can conceivably be affected either by a decrease in the concentration of PGs or by a change in their macromolecular composition. The influence of change in the macromolecular composition on the morphogenesis has been elucidated in various organ culture systems [14, 17, 19, 22]. In the present investigation, mainly a decreased biosynthesis along with minor changes in the nature of PGs synthesized were observed. Although there was an absolute decrease in PGs, a relative increase in the synthesis of chondroitin sulfate-proteoglycan (CS-PG) in the tissue fraction was observed. Along with these changes, the media PGs/GAGs synthesized under the influence of PAN were of a relatively smaller size. In view of the findings indicated above, it seems that it is the absolute decrease in the overall synthesis of PGs, rather than the change in their macromolecular composition which resulted in the perturbed morphogenesis of the kidney. The relative increase in the synthesis of CS-PG may be related to the sparing of mesenchymal cells by PAN. Presumably, these cells like mesangial cells synthesize large amounts of CS-PG [39–41]. Thus, the absolute decrease in the synthesis of PGs is most likely due to the selective effects of PAN on the epithelial cells which primarily synthesize heparan sulfate-proteoglycans (HS-PG). Such selective effects of PAN on the epithelial cells as well as on the biosynthesis of HS-PGs in in vitro culture system has been described in our previous publication [9].

In keeping with our previous observations [9], the results obtained in this investigation suggest that PAN interferes in the metabolism of HS-PG by affecting the synthesis of its core peptide. Absence of any major change in the macromolecular composition of HS-PG, synthesized by metanephric tissues, would seem to indicate that PAN interferes in the protein synthesis while leaving the post-translational processes unaffected. Whether the PAN affected the transcriptional events, such as tRNA synthesis, in a manner similar to that of puromycin hydrochloride in this metanephric system remains to be investigated. However, the data from our limited experiments with puromycin hydrochloride indicate no significant effect on the morphogenesis of the kidney or proteoglycans (unpublished results).

Other interesting effects of PAN, observed in this investigation, pertain to the interference in synthesis of the core peptide of the PGs. We previously reported that PAN affects the synthesis of core peptide of a relatively smaller-sized proteoglycan ($M_r \sim 1.5 \times 10^5$) [9]. Along these lines, the histochemical, immunofluorescent and immunopreciptation data included in this investigation indicate that PAN is also capable of perturbing the synthesis of the core peptide of a relatively higher molecular weight PG ($M_r > 2.5 \times 10^6$). The nature of this large molecular weight proteoglycan is somewhat controversial. It could be similar, in certain respects, to the large molecular weight basement membrane heparan sulfate-proteoglycan [42, 43]. However, its nucleotide sequence homology and immunological cross reactivities with laminin [43] preclude one from further correlative discussion at this point. In regard to the large molecular weight embryonic proteoglycan, Platt et al reported it to be primarily composed of chondroitin sulfate chains, presumably having immunologic cross reactivities with core peptide of heparan sulfate proteoglycan isolated from EHS-sarcoma [22]. In our studies, it seems to be predominantly made up of heparan sulfate GAG chains [14]. These discrepancies may be related to differences in the culture conditions used. For instance, inclusion of insulin and triiodothyronine can significantly influence posttranslational modifications of macromolecular composition of the proteoglycans. In this regard, we have indeed shown that insulin-like growth factor (IGF-1) selectively stimulates the synthesis of chondroitin sulfate-proteoglycans by various cell types of the glomerulus [44]. Lastly, a comment can be made in regard to the selective effect of the PAN on the core peptide of the proteoglycans. Our immunofluorescent and immunoprecipitation data indicate that other high molecular weight connective tissue proteins, that is, laminin and type IV collagen, were relatively unaffected. These findings go along with our previous data of glomerular epithelial cell cultures, where PAN selectively perturbed biosynthesis of PGs while leaving other glycoproteins, such as Fx1A (a tubular brush protein), unaffected [9].

In summary, the fact that the PAN-induced metanephric dedifferentiation was associated with biosynthetic changes in proteoglycans suggests that these protein-polysaccharide complexes are highly relevant macromolecules in various differentiation processes in mammalian tissues.

Acknowledgments

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